Molecular aspects of HNPCC and identification of mutation carriers

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Chapter 4

BRAF is not involved in the colorectal tumorigenesis of HNPCC

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ABSTRACT

Background: Recently it was shown that the oncogenic activation of *BRAF*, a member of the RAS/RAF family of kinases, by the V600E mutation is characteristic for sporadic colon tumours with microsatellite instability. Further, it was shown to associate with the silencing of the mismatch repair (MMR) gene *MLH1* by hypermethylation. Moreover, *BRAF* mutations proved to be absent in tumours from hereditary nonpolyposis colorectal cancer syndrome (HNPCC) families with germline mutations in the MMR genes *MLH1* and *MSH2*. These data suggest that the oncogenic activation of *BRAF* is involved only in sporadic colorectal tumorigenesis. In order to further support this hypothesis, we have extended the analysis of the *BRAF* gene to a different subset of HNPCC families without germline mutations in *MLH1* and *MSH2*. **Methods:** *BRAF*-V600E mutations were analysed by automatic sequencing in 38 tumours from HNPCC families with germline mutations in the *MSH6* gene and also in HNPCC (suspected) families that do not have mutations in the MMR genes *MLH1*, *MSH2* and *MSH6*. All patients belong to different families. **Results:** No mutations were detected in 14 tumours from HNPCC patients with germline mutations in *MSH6*. Further, no mutations of *BRAF* were found in tumours from 23 MMR negative families, from which 13 fulfilled the Amsterdam criteria (HNPCC) and 10 were suspected for HNPCC as they were positive for the Bethesda criteria. **Conclusion:** Overall, our data reinforce the concept that *BRAF* is not involved in the colorectal tumorigenesis of HNPCC. The detection of a positive BRAF-*V600E* mutation in a colorectal cancer suggests a sporadic origin of the disease and the absence of germline alterations of *MLH1*, *MSH2* and also of *MSH6*. These findings have a potential impact in the genetic testing for HNPCC diagnostics and suggest a potential use of *BRAF* as exclusion criterion for HNPCC or as a molecular marker of sporadic cancer.

INTRODUCTION

Activation of the RAS/RAF pathway is the most common oncogenic event in colorectal tumorigenesis. In addition to mutations in *KRAS*, a V600E hotspot mutation in the exon 15 of *BRAF*, a member of the RAF family of kinases, has been recently reported in colorectal tumours with high microsatellite instability (MSI-H) and was found associated to a defective mismatch repair (MMR) system.[1-3] Further, it was shown that the *BRAF*-V600E mutation

(previously named V599E) is mainly found in proximal colorectal tumours in which the MSI-H phenotype is caused by hypermethylation of the MMR gene *MLH1*.[4,5] Tumours arising in the hereditary nonpolyposis colorectal cancer syndrome (HNPCC) are preferentially found in the proximal colon and show MSI due to germline defects in genes from the MMR system, mainly *MLH1*, *MSH2* and *MSH6*.[6,7] Therefore, mutations in *BRAF,* as an alternative oncogenic event to *KRAS* activation, were expected to play a role in the tumorigenesis of HNPCC. However, recent data have provided evidence that *BRAF* is not involved in tumours from HNPCC patients with germline mutations in *MLH1* and *MSH2*, suggesting that the oncogenic capability of *BRAF* in MSI colon cancer might be somehow related to the epigenetic mechanisms involved in the inactivation of the *MLH1* gene and not to the germline MMR defect.[4,8,9] Owing to its absence in the *MLH1* and *MSH2* germline mutation-positive families, a potential use of *BRAF*-V600E in the molecular diagnostics of HNPCC has been suggested.[8,9] Nonetheless, approximately 5% of cases show germline mutations in the *MSH6* gene and about half of all families clinically defined as HNPCC do not have mutations in any of the known MMR genes, and for these cases it is yet not known whether *BRAF*-V600E might play a role in tumorigenesis.[7,10-12] Here we report data that support the hypothesis that *BRAF* is not involved in the tumorigenesis of the HNPCC-related tumours considering HNPCC as diagnosed on both molecular, by the presence of a germline mutation in a MMR gene, or on clinical ground, fulfilling the revised Amsterdam criteria. Our data suggest a potential use of *BRAF*-V600E as the exclusion criterion for HNPCC or as a molecular marker of sporadic cancer.

MATERIAL AND METHODS

Tumour specimens

A total of 33 colorectal tumours were obtained from HNPCC patients with germline alterations in the *MSH6* gene (n=10) and from HNPCC families negative for germline mutations in the MMR genes *MLH1*, *MSH2* or *MSH6* (n=22). Two extracolonic tumours from two of these patients were also analysed as well as 3 extracolonic tumours from 3 additional patients (Table 1). Tumours were obtained from the University Hospital Groningen (Groningen, The Netherlands), Sapporo Medical University (Sapporo, Japan) and also from several different hospitals in Finland. Sample collection was carried out in accordance with

the previously established ethical protocols from each one of the participating institutions, and the respective ethics committees approved the study. Genomic DNA was extracted with phenol-chloroform according to standard procedures. Microsatellite instability was analysed according to the international criteria for the determination of microsatellite instability, using various panels of dinucleotide and mononucleotide repeat sequences as previously described.[13] Accordingly, tumours were classified as MSI-H or MSI-low (MSI-L) when showing high or low levels of instability, respectively.

Tumour samples had been analysed previously for germline mutations in the MLH1, MSH2 and MSH6 genes by several laboratory routines and mutations verified by automatic sequencing. In some cases, large deletions of *MLH1* and *MSH2* were detected by Southern blotting or using alternative strategies.[14,15] Immunohistochemical stainings for MLH1 (clone G168-728, BD Pharmingen, San Diego, CA, USA), MSH2 (clone FE11, Calbiochem, San Diego, CA, USA), MSH6 (clone 44, Transduction Laboratories, Lexington, KY, USA), PMS1 (sc-615, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and PMS2 (sc-618, Santa Cruz Biotechnology Inc.) were also performed.

Mutational analysis of the BRAF-V600E hotspot.

The analysis of *BRAF* was performed by automatic sequencing. The fragment encompassing exon 15 was amplified by PCR in all carcinoma samples. Primer sequences and PCR conditions were based on those reported previously.[1] Genomic DNA (25 - 100 ng) was amplified by PCR using the following cycling conditions: 30 seconds at 94ºC, 30 seconds at 60ºC, and 45 seconds at 72ºC for 35 cycles. PCR products were purified and sequenced on an ABI Prism 377 Automatic sequencer (Perkin-Elmer, Foster City, CA) using the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin-Elmer).

Two sided Fisher's exact test was used for statistical analysis.

Table 1. Features of HNPCC tumours found negative for the *BRAF*-V600E mutation

Patient	MMR[1]	Mutation	Location	MSI	Age	Criteria[2]
Y88	MSH6	truncating (3263insT)	transverse	MSI-H	38	-
Y701	MSH6	truncating (650insT)	rectum	MSI-H	53	-
Y708[3]	MSH6	truncating (2672delT;2674delT)	descendent	MSI-H	55	-
Y725	MSH6	truncating (650insT)	left-sided[4]	MSI-H	83	+
Y1	MSH6	truncating (Glu1258X)	rectum	MSI-L	55	-
Y37	MSH6	truncating (650insT)	descendent	MSI-L	59	-
Y241	MSH6	missense (Ala355Val)	descendent	MSI-H	65	-
Y105	MSH6	missense (Ile725Met)	transverse	MSI-L	36	-
Y243	MSH6	missense (Gln522Arg)	rectum	MSI-L	43	-
Y319	MSH6	missense (Pro1087Ser)	rectum	MSI-L	39	-
Y194	-	-	rectum	MSI-H	58	+
Y249	-	-	transverse	MSI-H	48	+
CCH1	-	-	ascendent	MSI-H	46	+
CCH2	-	-	cecum	MSI-H	47	+
CCH3	-	-	descendent	MSI-H	49	+
CCH4	-	-	transverse	MSI-H	38	+
CCH5	-	-	cecum	MSI-H	42	+
CCH6	-	-	descendent	MSI-H	47	+
CCH7	-	-	ascendent	MSI-H	31	+
CCH8	-	-	cecum	MSI-H	45	+
CCH9	-	-	cecum	MSI-H	43	-
CCH11	-	-	transverse	MSI-H	50	-
CCH12	-	-	cecum	MSI-H	46	-
CCH14	-	-	transverse	MSI-H	48	-
CCH15	-	-	cecum	MSI-H	55	-
CCH16	-	-	cecum	MSI-H	44	-
CCF265	-	-	ascendent	MSI-H	82	-
CCF910	-	-	cecum	MSI-H	81	-
Y74	-	-	rectosigmoid	MSI-L	48	+
Y168	-	-	sigmoid	MSI-L	35	+
CCH10	-	-	ascendent	MSI-L	62	-
CCH13	-	-	ascendent	MSI-L	44	-
Other tumours						
Y1	MSH6	truncating (Glu1258X)	pyelum	MSI-H	63	-
Y37	MSH6	truncating (650insT)	endometrium	MSI-L	65	-
Y605	MSH6	truncating (650insT)	endometrium	MSI-L	46	+
Y751	MSH6	truncating (650insT)	duodenum	MSI-L	51	-
Y77	-	-	duodenum	MSI-H	54	+

1: Defective MMR gene. Negative cases do not show mutations in *MLH1*, *MSH2* or *MSH6* genes. 2: Positive cases fulfil the Amsterdam criteria and negative cases are from families showing an HNPCC-like pedigree in which at least one first degree relative is affected by an early onset colorectal cancer. 3: This patient also showed a synchronous MSI-H tumour in the cecum that was positive for *BRAF*-V600E and showed *MLH1* hypermethylation. 4: this tumour is left-sided although its exact location is unknown.

RESULTS AND DISCUSSION

We have analysed 38 tumours from two different subsets of HNPCC (suspected) patients: those that harbour an *MSH6* germline defect, and patients from families fulfilling the clinical criteria but in whom no *MLH1*, *MSH2* or *MSH6* germline mutations could be identified (see Table 1). All patients belong to different families. As shown in Table 1, the analysed cases either fulfilled the Amsterdam criteria or were positive for specific criteria from the Bethesda guidelines (HNPCC-like).[11,16,17] The indexed patients of these HNPCC-like families have early onset colorectal cancer and at least a first-degree relative with a HNPCC-related tumour.[17]

We have recently suggested the introduction of the *BRAF*-V600E mutation screening in the molecular diagnostic protocols for HNPCC as a low-cost effective strategy that allows simplifying the genetic testing of HNPCC patients.[9] The reported absence of V600E mutations in HNPCC cases that harbour germline mutations in the *MLH1* and *MSH2* genes, predicted a potential use of *BRAF* as a pre-screening tool in HNPCC as only *BRAF*-V600E-negative cases need to be screened for mutations in the MMR genes *MLH1* and *MSH2*.[4,8,9] Further, it suggests that *BRAF* mutations are not involved in HNPCC tumorigenesis, but are restricted to the sporadic cases. However, it is unknown yet whether *BRAF* could be involved in HNPCC tumours without germline mutations in *MLH1* and *MSH2*. In fact, almost half of the HNPCC families show no mutations in the MMR genes and about 5% of cases are due to germline mutations of the *MSH6* gene.[7,10-12,18]

To answer these questions, *BRAF*-V600E mutations were screened in 12 colon tumours derived from patients from HNPCC families that fulfilled the Amsterdam criteria but did not show germline mutations in *MLH1*, *MSH2* or *MSH6*. In none of these tumours, the *BRAF*-V600E mutations were identified. In all, 10 of these tumours were classified as MSI-H and two as MSI-L, according to the international Bethesda criteria.[13] An additional MSI-H tumour from the duodenum of an HNPCC family positive for Amsterdam criteria was also found negative for *BRAF* mutations. Furthermore, no mutations were detected in 10 colorectal tumours, eight MSI-H and two MSI-L, respectively, from suspected HNPCC families not fulfilling the Amsterdam criteria, but being positive for clinical criteria suggestive of familial cancer. Also, we have extended our analysis to colon tumours, five MSI-H and five MSI-L, from 10 HNPCC(like) families showing germline mutations in *MSH6*. Of these families, six

proved to have truncating and four missense mutations of this gene (Table 1). Regarding these families, one was positive for Amsterdam criteria and nine were suspected HNPCC, according to the clinical criteria above described. We did not detect *BRAF*-V600E mutations in these cases. Two additional tumours from the endometrium and pyelum from two patients of these families, as well as two extracolonic tumours from the duodenum and endometrium from two additional families showing germline truncating mutations in *MSH6*, were also negative for *BRAF* mutations (Table 1).

Interestingly, in one of the analysed patients with a truncating *MSH6* germline mutation, a synchronous MSI-H colorectal cancer in the cecum was found positive for the *BRAF*-V600E mutation. This tumour, however, showed an absence of MLH1 by immunohistochemistry (IH) and hypermethylation of the *MLH1* promoter. This finding is best explained by the significant reported association of *BRAF*-V600E with the hypermethylation of *MLH1* as seen in a high frequency of sporadic tumours, a phenomenon not directly linked to the germline mutation of *MSH6*.[4,5]

Overall, we did not detect significant *BRAF*-V600E mutations in any of these HNPCC subsets, reinforcing the idea that *BRAF* is not involved in HNPCC tumorigenesis, independently of the MMR gene defect and independent of the presence of high or low MSI phenotypes. These results suggest a potential use of the *BRAF* mutation as a marker of sporadic colorectal cancer.

Therefore, we suggest that detection of a positive *BRAF*-V600E mutation in a colorectal cancer is most likely suggesting a sporadic origin of the disease and the absence of germline alterations of *MLH1*, *MSH2* and also of *MSH6*. Further, these findings might also have a potential impact in the gene testing for HNPCC diagnostics.

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